PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Handfield, et al.	)	
	)	Group Art Unit: 1646
Serial No.: 09/995,493	)	
	)	Examiner: Baskar, P.
Filed: November 28, 2001	)	
	)	Docket No.: 01-662

For: **Identification of *Actinobacillus actinomycetemcomitans* antigens for use in the diagnosis, treatment and monitoring of periodontal disease**

DECLARATION

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Dr. Martin Handfield, am a named inventor of U.S. Patent Application Serial No. 09/995,493, filed on November 28, 2001 ("the Patent Application").

2. Experimental data clearly demonstrate that IVIAT proteins tested to date in *A. actinomycetemcomitans* and other systems are indeed immunogenic, that they result in many cases in seroconversion with patients infected with a pathogen, and that proteins discovered with IVIAT are involved in pathogenesis (not simply immunogenic in patients). Furthermore, in many cases, these proteins would not be otherwise recognized as involved in human disease without the use of IVIAT methodology. Experiments and data supporting these statements are described below.

3. A key experiment, which was performed under my supervision, involved probing a purified IVIAT protein (which comprised SEQ ID NO:52) with sera from individual patients (as opposed to pooled sera, as was used in Handfield *et al.*, Trends in Microbiology, 8:336 (2000)). A Western blot of SEQ ID NO:52 was probed with sera from 20 patients vs sera from 10 control patients. A Western blotting approach was used

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to determine if sera from periodontitis patients possessed higher reactivity with the selected IVIAT protein than sera from control patients. Densitometric and statistical analyses (Student's T-test) of reactive bands appearing in the Western blot demonstrated that patient sera had, on average, significantly ($p<0.05$) greater reactivity with the IVIAT protein comprising SEQ ID NO:52. This experiment demonstrated that the IVIAT protein is not equally immunoreactive with all patients and controls, which suggests that if there was cross reactivity, the source would not be something that is common to all patients (i.e., that constitutes a normal component of human proteins). This data also demonstrated that this protein is significantly ($P < 0.05$) more immunoreactive in patients than the level of immunoreactivity (or background level of cross reactivity) seen in control patients. This experiment also suggests that most patients do indeed encounter SEQ ID NO:52 during infection, which confirms the data found with the original dot-blots and immunofluorescence experiments described in Handfield *et al.* A low level of background reactivity was detected and was anticipated since *A. actinomycetemcomitans* is part of the human flora.

4. The open reading frame encoding SEQ ID NO:52 was induced intracellularly using an *in vitro* experimental model of infection. HeLa cells were infected with wild-type *A. actinomycetemcomitans,* strain VT1169, and specific gene expression assayed using real-time polymerase-chain reaction. Controls for this experiment included *lktA* encoding the well-studied leucotoxin gene, which is expressed during *in vitro* growth of the pathogen (Hritz *et al.,* Infect. Immun. 64:2724-9 (1996)). Under the infection conditions used, virtually every HeLa cell was infected at a level of 1-5 CFU per HeLa cell, or 0.1-0.5% of the inoculum, which is consistent with previously reported data (Meyer *et al.*, Infect. Immun. 59:2719-26. (1991)). The template amount used in the assay was normalized using the total count of viable bacteria (CFU) as a reference point, and done in duplicate in at least two independent experiments. The transcription of the open reading frame encoding SEQ ID NO:52 was significantly ($p<0.05$) increased in HeLa cells as compared to bacteria grown extracellularly. For this transcript, the induction level was 4-fold. Expression of *lkt*A was not significantly ($p=0.3$) different during invasion of HeLa cells compared to *in vitro* grown cells. Interestingly, the

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expression the rRNA was also induced 5 fold intracellularly, which is consistent with the increased growth rate observed by others (Fives-Taylor *et al.*, Periodontol. 2000 20: 136-167 (1999)). This data suggests that the open reading frame comprising SEQ ID NO:52 is in fact regulated by environmental cues found intracellularly.

5. Similar findings regarding IVIAT proteins were recently reported in our collaborative work on *Vibrio cholera* (Hang *et al*, PNAS 100:8508 (2003)) (copy attached). Of most importance in that report, two IVIAT antigens discovered resulted in seroconversion in infected patients as compared to uninfected controls (Hang *et al.*, Fig 3.). Proteins identified using IVIAT methodology do not, therefore, appear to result from non-specific cross reactivity that could be found in human sera, or from cross reactivity resulting from an immune response initially raised against other bacterial antigens since the immunoreactivity is variable and greater in patients versus controls, and this it true for a majority of IVIAT antigens discovered thus far and in every system tested. Our work on *V. cholerae* allowed the recovery of 16 antigens induced specifically in humans. Of special interest was the discovery of PilA in that IVIAT screen. That protein was found in convalescent patients and its expression appears to be unique to the human infection and impossible to study in current animal models of infection. The involvement of this antigen in the pathogenesis of *Vibrio* has not otherwise been found using current alternative technologies, which suggests that IVIAT truly recovers unique targets for diagnostic purposes that are specific to human infections and that could not be found otherwise.

6. Further evidence that IVIAT proteins are specifically expressed during infection and that, therefore, the immunoreactivity observed is real and not due to specific or non-specific cross reactivity, has been reported in a number of publications. In particular, our work on *Candida albicans* (Cheng *et al.*, 48 Mol. Microbiol. 1275 (2003)) (copy attached) identified ten antigens with IVIAT. All ten antigens were unambiguously specifically expressed in human thrush but not during *in vitro* cultivation of the pathogen. The disruption of one of the IVIAT genes resulted in attenuated virulence in an animal model of candidal infection.

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7. Similar findings were reported on our work in *Vibrio vulnificus* (Kim et al., Infect. Immun. 71:5461 (2003)) (copy attached). In that project, 12 IVIAT antigens were found in this organism and the inactivation of a number of them resulted in a decreased viability, cytotoxiticy or virulence in an animal model of infection.

8. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon."

Date: 2/10/04

By: _____
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